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The Role of Arp2/3 in Ring Canal Development in *Drosophila melanogaster*

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The Role of Arp2/3 in Ring Canal Development in *Drosophila melanogaster*

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Butler University

The Role of Arp2/3 in Ring Canal Development in *Drosophila melanogaster*

A Thesis

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Marina A. Tipold

Table of Contents

Abstract	4
Introduction	5-9
Infertility	5
Intercellular Bridges and Gametogenesis.....	5-6
<i>Drosophila melanogaster</i> oogenesis as a model to study intercellular bridges.....	6-8
The Arp2/3 Complex is Necessary for Intercellular Bridge Structure.....	8-9
Misshapen is a Novel Ring Canal Protein.....	9
Background on Methods.....	10-12
UAS/GAL4 and FLP/FRT System.....	10-11
Arp2/3 Inhibitor Treatment.....	11-12
Thesis Research and Hypothesis	12
Materials and Methods	13-17
<i>Drosophila Melanogaster</i> Environmental Conditions	13
<i>Drosophila Melanogaster</i> Genetics	14
Dissection and staining of ovarian tissue	14-15
Arp2/3 Inhibitor Treatments (CK-666, CK-689).....	15
Microscope Imaging and Analysis	16
Quantification and Statistical Analysis.....	16-17
Results	18-26
Deletion or mutation of Arp2/3 complex members leads to smaller ring canals..	18-21
Mutation of Arp2/3 complex members also alters ring canal size.	21-22
Acute inhibition of Arp2/3 functions leads to decrease in ring canal size.	23-24
Msn and Arp2/3 lead to ring canal growth.	24-26
Discussion	27-32
The Arp2/3 protein complex is required for ring canal growth.	27-30
Arp2/3 plays a role in ring canal development along with Msn.	30-32
Conclusion	33
References	34-36

Abstract

Infertility and impaired fecundity affect 8.2 million women in the United States.

Intercellular bridges are essential to the proper formation of germ cells in many organisms; therefore, learning more about how they are formed and regulated during the formation of sperm and eggs could provide insight into how defects in their structure can impact fertility. Intercellular bridges are actin-rich structures that connect developing germ cells to each other and allow the transfer of materials. I used the development of the fruit fly egg as a model system to study formation and regulation of intercellular bridges. Specifically, I investigated the role of an actin nucleating protein complex located at the ring canals, Arp2/3, in the formation and expansion of these structures. The objective of this work is to characterize the effect of depletion, mutation, or inhibition of the Arp2/3 complex on the structure and growth of these intercellular bridges, called ring canals. Because ring canals are rich in actin and actin-binding proteins, I predicted that if Arp2/3 levels are altered, then ring canal formation and/or growth will be affected. When levels of Arp2/3 complex proteins were reduced or if the complex was chemically inhibited, ring canals are significantly smaller than in controls; this difference was more pronounced in later stages of oogenesis (stages 7-10b), suggesting Arp2/3 plays a role in ring canal structure and stability in later stages of oogenesis. Because these structures are found in organisms from insects to humans, research on the Arp2/3 complex provides valuable understanding of intercellular bridge structure and function that can be applied to higher organisms and our understanding of infertility.

Introduction

Infertility

Reproductive disorders represent a significant issue within the healthcare field. Infertility and impaired fecundity affect 8.2 million women in the United States alone (1). In order to open doors to possible treatment methods, it is important to obtain more knowledge on the mechanism of normal germ cell development. Infertility can be caused by a number of different underlying defects, such as uterine polyps, irregular menstrual cycles, and adhesions on the ovaries, which make it difficult for the egg to transfer to the fallopian tube. Pelvic adhesions, such as ovarian cysts, are also known to be one of the primary causes of infertility (2). Although all of these conditions affect fertility, they primarily impact the stages after fertilization.

However, in many cases of infertility, the underlying cause is not known. One possible explanation for these cases is that infertility could arise from defects in gamete formation, which must occur successfully before fertilization can occur. Therefore, studying the normal processes that are involved in gametogenesis could provide important insight into understanding the causes behind and developing treatments for infertility.

Intercellular Bridges and Gametogenesis

One of the key steps in reproduction is the formation of gametes, or gametogenesis, in which haploid sperm and eggs are generated. In most organisms that reproduce sexually, the developing gametes go through a stage where they are

connected to each other or to supporting cells through intercellular bridges (3).

Intercellular bridges are important to allow developing germ cells to share cytoplasmic contents (mRNA, organelles, proteins) with either other germ cells or supporting cells.

Germline intercellular bridges are conserved cellular structures that often expand during development (3). These structures, called ring canals in *Drosophila*, are found in organisms from the fruit fly to mammals; however, their sizes vary significantly. Vertebrate species such as mice and humans have clusters of connected germ cells during fetal development. These clusters of germ cells are not present through adulthood, making studying germline intercellular bridges more difficult in some species (4). Due to the difficulty in studying intercellular bridges during oogenesis in vertebrates, the use of an invertebrate model system is necessary.

Defects in intercellular bridge structure or stability can lead to infertility (5). For example, in mice, intercellular bridges mediate cytoplasmic connections between developing spermatids. When intercellular bridges have insufficient expansion or become collapsed through development, the mitotic divisions are inhibited. The improper transfer of materials leads to a loss of spermatid function leading to infertility (6). Therefore, learning more about the structure and expansion of ring canals could provide important insight into the causes of infertility.

***Drosophila melanogaster* oogenesis as a model to study intercellular bridges**

The developing fruit fly egg is an excellent invertebrate model system in which to study intercellular bridge formation, structure, and growth. The *Drosophila melanogaster* egg develops from an organ-like structure called an egg chamber. The egg

chamber contains a central cluster of germ cells (15 nurse cells and an oocyte), which is encapsulated by somatic cells called follicle cells (7). It progresses through fourteen stages of development before giving rise to a mature egg (3). The egg chambers are formed within a structure called the germarium, which contains somatic and germline stem cells. Egg chamber formation begins with the division of a germline stem cell (GSC), which gives rise to two daughter cells – another germline stem cell and a cystoblast cell (CB). The cystoblast cell goes through four rounds of mitosis followed by incomplete cytokinesis to generate a 16- cell cyst of interconnected cells (3,8). Of the 16 cells, one cell will form the oocyte and the other 15 cells will be supporting cells called nurse cells. The oocyte and nurse cells are all connected to each other through intercellular bridges called ring canals, which are rich in filamentous actin and actin binding proteins (9).

The ring canals allow a cytoplasmic connection to be maintained between the nurse cells and the developing oocyte, and this connection is essential for fertility (9). The oocyte remains transcriptionally inactive during oogenesis, so the ring canals allow for materials such as proteins, mRNA, and even organelles to pass from the nurse cells to the oocyte. During the early stages of oogenesis (prior to stage 11), this transfer is slow and mediated by microtubule motor proteins. However, at stage 11, there is a rapid event called “nurse cell dumping” in which the nurse cells transfer all of their contents to the oocyte, which doubles in volume over a 30-minute period (7). In order to accommodate these transfer events, the ring canals must grow in size significantly (from $\sim 0.5\ \mu\text{m}$ in diameter to $\sim 10\ \mu\text{m}$ in diameter; 10). The growth in ring canal

diameter correlates with a change in the number and organization of actin filaments present within the ring canal (11). Early in ring canal expansion, the number of actin filaments increases from ~ 50 filaments to around 700 filaments, coincident with an expansion in diameter to about 5 μ m. However, beginning around stage 5 of oogenesis, the number of filaments remains constant while the diameter continues to expand from ~5-10 μ m (12). This suggests that changes in the rate of actin filament formation and organization are necessary to promote ring canal expansion.

The Arp2/3 Complex is Necessary for Intercellular Bridge Structure and Expansion

In the egg chamber, a number of proteins have been shown to localize to the ring canals and promote their formation, structural integrity, or expansion. Many of the proteins that localize to the ring canals can bind to and/or bundle actin filaments (12). For example, filamin and Kelch, two F-actin binding proteins located at the ring canal, play a role in altering the branching F-actin network to form bundles throughout development (12). In addition, the seven-subunit complex, Arp2/3, is also found at the ring canals, and it plays a role in formation of branched actin networks through nucleating new actin filaments from existing actin structures (13). Specifically, two of the seven protein subunits in the Arp2/3 complex, Arp2 and Arp3 (14), and the activator of the Arp2/3 complex, SCAR, have been shown to localize to the germline ring canals (15). Further, mutations in Arp2, Arp3, or SCAR lead to inhibited growth and collapse in ring canal structure and disruption of nurse cell dumping (12). However, it is unclear how Arp2/3 regulates the growth and stability of ring canals in the egg chambers of *Drosophila melanogaster*. Therefore, the goal of this work is to characterize how Arp2/3

promotes ring canal growth and stability, as well as begin to investigate the mechanisms by which the activity of the Arp2/3 complex is controlled at the ring canals. The insights gained from research on the Arp2/3 complex in the egg chamber will provide valuable understanding of intercellular bridge structure and function that can be applied to higher organisms where Arp2/3 function is conserved.

Misshapen is a Novel Ring Canal Protein

Although it was previously shown that the Arp2/3 complex is necessary for proper ring canal growth and stability, the mechanisms controlling Arp2/3 activity in the germline are not known. Recently, the mammalian protein, NIK, has been shown to activate the Arp2/3 complex by phosphorylating an Arp2/3 subunit (16). Misshapen (Msn), the *Drosophila* homolog of NIK, localizes to the ring canals and plays a role in their growth and stability in the egg chambers of *Drosophila melanogaster*. Preliminary data from our lab show that when Msn is depleted, ring canal growth, expansion, and stability are disrupted. Therefore, our model is that Msn could be promoting Arp2/3 activity at the ring canals to promote their expansion and maintain a stable intercellular connection between the nurse cells and the developing oocyte.

Background on Methods

UAS/GAL4 and FLP/FRT System

The UAS/GAL4 system was used in combination with RNA interference (RNAi) to deplete the germline of Arp2/3 subunits or Msn in *Drosophila melanogaster*. The UAS/GAL4 system is a binary system allows the tissue specific expression of transgenes of interest. Crosses are set up between a transgenic line that contains the GAL4 transcriptional activator controlled by a tissue specific promoter (the GAL4 “driver” line) and a line that contains a transgene of interest downstream of an upstream activating sequence (UAS). In the progeny of the cross, the GAL4 protein will be expressed in the tissue of interest, and it will bind to the UAS sequence and promote expression of the downstream transgene (17). In these experiments, the driver line will express the GAL4 protein only in the germline (nanos-GAL4 or a triple GAL4 driver line, otu-GAL4; nanos-GAL4; nanos-GAL4). These germline drivers will be crossed to stocks that contain UAS-ArpC2-RNAi and/or UAS-Msn-RNAi transgenes.

RNAi is a technique used to deplete a target protein by degrading the corresponding mRNA. A short hairpin RNA complementary to the mRNA of interest is introduced and the Dicer enzyme cleaves the short hairpin RNA in to smaller pieces, around 20 nucleotides long, allowing them to become part of the RNA-induced silencing complex (RISC) complex. Once integrated into the RISC complex, the siRNA can bind to the mRNA of interest, yielding desired degradation (18).

The FLP-FRT system was used to generate mosaic egg chambers to evaluate the effect of loss of Arp2/3 function. Mosaic egg chambers contain germ cells that are

homozygous for recessive mutations in members of the Arp2/3 complex genes in an otherwise heterozygous mutant background. The mutations are present on chromosome arms that contain FRT recombination sites. Stocks containing the mutant chromosomes are crossed to other stocks that contain a FLP recombinase enzyme controlled by a heat shock promoter in addition to a homologous chromosome containing a visible marker downstream of the FRT site, such as GFP. Upon heat shock treatment at 37°C, the FLP recombinase is activated and induces mitotic recombination between the two FRT-containing chromosomes. During anaphase, when the sister chromatids separate, this can generate homozygous mutant cells next to homozygous wild type (GFP-marked) cells. The progeny from these two daughter cells will continue to divide and generate mosaic tissue (19). Therefore, the FLP-FRT system can be used to generate egg chambers that contain germ cells that are homozygous for mutations in the Arp2/3 complex members.

Arp2/3 Inhibitor Treatment

Small molecule inhibitors can work as a drug to inhibit a protein or complex of interest, in this study the Arp2/3 protein complex. The small molecule inhibitor, CK-666, binds to the Arp2/3 protein complex. When binding to the protein complex, f-actin nucleation is inhibited. CK-666 blocks the movement of two of the seven Arp2/3 subunits into the active conformation, rendering the Arp2/3 protein complex inactive (20). CK-666 is a commercially available compound (EMD Millipore). In addition to CK-666, a control molecule was used, CK-689. The CK-689 control molecule has a similar structure to CK-666, but it does not inhibit the Arp2/3 complex (20). Using CK-666, an

acute chemical treatment can be performed on the egg chambers in order to determine whether Arp2/3 is continuously required during oogenesis to maintain ring canal structure or promote growth.

Thesis Research and Hypothesis

Research on the Arp2/3 complex provides valuable understanding of intercellular bridge structure and function that can be applied to higher organisms and our understanding of infertility. Intercellular bridges are essential to the proper formation of germ cells in many organisms; therefore, learning more about how they are formed and regulated during the formation of sperm and eggs could provide insight into how defects in their structure can impact fertility. The development of the fruit fly egg was used as a model system to study the role of the Arp2/3 complex in formation and regulation of intercellular bridges. The objective of this work was to characterize the effect of depletion, mutation, or inhibition of the Arp2/3 complex on the structure and growth of ring canals. Because ring canals are rich in actin and actin-binding proteins, I predicted that if Arp2/3 levels are altered, then ring canal formation and/or growth would be affected. Here, I show that when the Arp2/3 protein complex activity is altered through depletion, mutation, or inhibition, ring canal formation and growth is disrupted, leading to smaller ring canals than seen in controls, which often collapse.

Materials and Methods

***Drosophila melanogaster* Environmental Conditions**

Drosophila melanogaster fly stocks were kept in vials with standard cornmeal molasses food (cornmeal, molasses, yeast, tegosept, and propionic acid) made using standard procedures. The fly stocks were stored in constant temperature incubators, keeping the flies at 25°C in order to maintain a generation time of 10-12 days. Genetic crosses were performed by placing male flies from one stock and virgin female flies from another stock into a vial with fresh yeast at 25°C. The adults used to set up the crosses were flipped into a new vial with food and fresh yeast every 4 days in order for the cross to be used for a longer period of time. To alter transgene expression in the ovaries, offspring from crosses were kept at varying temperatures (25° and 29°) and time periods (48-72 hours) before dissection. Conditions for each experiment are listed in Table 1.

Table 1. Experimental genotypes and conditions for transgene expression.

Genotype	Temperature at which cross was raised	Heat Shock	Temperature of females on yeast	Time of females on yeast
	°C	✓	°C	h
UAS-ArpC2-RNAi x MTD-GAL4	25		29	48-72
w ¹¹¹⁸ x MTD-GAL4	25		29	48-72
UAS-ArpC2-RNAi x otu-nos-GAL(111)	25		29	48
w ¹¹¹⁸ x out-nos-GAL(111)	25		29	48
UAS-msn-RNAi x MTD-GAL4	25		29	48
w ¹¹¹⁸ x MTD-GAL4	25		29	48
UAS-ArpC2-RNAi; UAS-Msn-RNAi x MTD-GAL4	25		29	48
w ¹¹¹⁸ x MTD-GAL4	25		29	48
hsFLP; ArpC1 ^{R337st} , FRT40/cyo x hsFLP; GFP, FRT40/cyo	25	✓	25	48
hsFLP; ArpC1 ^{Q25st} , FRT40/cyo x hsFLP; GFP, FRT40/cyo	25	✓	25	48
Arp3 ^{383F} , FRT80 x hsFLP; TubP-GAL4, GFP, GAL80, FRT80	25	✓	25	48
Arp3 ^{515FC} , FRT80 x hsFLP; TubP-GAL4, GFP, GAL80, FRT80	25	✓	25	48

***Drosophila melanogaster* genetics**

ArpC2 and/or Msn was depleted from the germline using the UAS/GAL4 system (17). UAS-ArpC2-RNAi (Bloomington #43132) and/or UAS-msn-RNAi (Bloomington #42518) were crossed to nanos-GAL4 (Bloomington #32563) or crossed to the maternal triple driver (otu-GAL4; nanos-GAL4; nanos-GAL4, Bloomington #31777), also referred to as MTD-GAL4. White-eyed, w^{1118} flies, are often used as the background when making transgenic flies. Therefore, these w^{1118} flies crossed to the germline drivers were used as a negative control. Crosses were raised at 25°C. Progeny from the crosses were selected for the appropriate genotype (against balancer chromosomes marked with dominant mutations that caused curly wings or short thick bristles/stubble) and aged on yeast in the presence of males for the time and temperature specified (Table 1).

Mosaic egg chambers were generated by crossing $hsFLP; ArpC1^{R377st}, FRT40/Cyo$ (Bloomington #9136) or $hsFLP; ArpC1^{Q25st}, FRT40/Cyo$ (Bloomington #9135) with $hsFLP; GFP, FRT40/Cyo. Arp3^{383F}, FRT80$ (Bloomington #39726) and $Arp3^{515FC}, FRT80$ (Bloomington #39727) were crossed to $hsFLP; TubP-GAL4, GFP, GAL80, FRT80. Arp3^{383F}$ is a point mutation changing a Cysteine to an Alanine. $Arp3^{515FC}$ is a nonsense mutation altering a Tryptophan. The $ArpC1^{R377st}$ mutation is a weaker allele than the $ArpC1^{Q25st}$ mutation, leading to a weaker phenotype. Each cross was heat shocked beginning at larval stages 1-2. The heat shock was a cycle of 2 hours at 37°C then 22 hours at 25°C. This cycle was repeated one more time and then the flies were maintained at 25°C.

Dissection and staining of ovarian tissue

Prior to dissection, ~10-12 female flies and 3-5 male flies were placed into a

fresh vial of fly food with live yeast in order to heighten egg production and enlarge the ovaries. Upon dissection, both control and experimental female flies were anesthetized using CO₂. Ovaries were dissected from the female flies using a stereomicroscope and forceps in Schneider's S2 media. Once dissected, ovaries were fixed in 4% formaldehyde solution (in PBS). The ovaries were washed (using PBS + 0.1-0.3% Triton X-100) and then stained with FITC-or TRITC-conjugated phalloidin (ECM Biosciences), DAPI (Life Technologies) and an Hts-RC antibody (DSHB, 1:20). After final stains and washes, the tissue was transferred onto a slide using a mounting solution (Slowfade Antifade; Invitrogen).

Arp2/3 inhibitor Treatments (CK-666, CK-869)

An acute chemical inhibition of Arp2/3 activity in egg chambers that are being cultured *ex vivo* was performed. Ovaries from female flies were dissected and individual ovarioles (strands of egg chambers at various stages of development) were isolated from the muscle sheath that they are enclosed in. The ovarioles were then transferred to media that contains either CK-666 (EMD Millipore, product #182515), inhibiting Arp2/3 complex activity, or into media that contains CK-689 (EMD Millipore, product #182517). Both the CK-666 and CK-689 were from 100µm stock solution of drug in DMSO (CK-666: 0.25mg drug and 0.844mL DMSO; CK-689: 0.25 drug and 1.076mL DMSO). Ovarioles were covered and incubated in either CK-666 or the control molecule for 120 minutes, and then the tissue was fixed, stained, imaged, and analyzed as described.

Microscope Imaging and Analysis

Images were collected using a Leica DMLB compound fluorescence microscope with an EXi Aqua cooled CCD camera (Qimaging), which has a motorized z-stage (Prior) and is controlled by Metamorph 7.7 software (Molecular Devices). The stained tissues were imaged and then analyzed using the computer program, *Fiji*. When imaging on the compound fluorescent microscope, z-stacks were taken through the thickness of the sample (with a z-step size of 2 μm) in order to see all of the ring canals within each egg chamber. By taking multiple images through a z-stack, ring canals that are not clear in one plane layer can be seen and measured in another plane. This allows visualization and analysis of all ring canals in a given egg chamber that was imaged. Using *Fiji*, images of each ovary were analyzed and the outer diameters of the ring canals were measured in pixels using a measuring tool within the program. The ring canals were measured from the Hts-RC stain. In order to standardize values when converting from pixels to microns, all samples were imaged using a 20x dry objective lens without binning. Data were collected for each ovary, recording the stage of each egg chamber measured, the size of the ring canals, and the number of collapsed ring canals.

Quantification and Statistical Analysis

To collect data of ring canal size in each egg chamber, the outer diameter of each ring canal was measured using a quantification tool in *Fiji*. The ring canal diameter was measured in pixels in the program, recorded, and later converted into microns. For each egg chamber, the stage was recorded based on morphology, the number of ring canals was counted, the size of measurable ring canals was measured, and the number of

collapsed ring canals was assessed. The average ring canal diameter for each condition at each developmental stage was calculated, and the standard deviation of the means was determined. In order to determine statistical significance between treatments and controls, a two-sample t-test was performed.

Results

Depletion or mutation of Arp2/3 Complex members leads to smaller ring canals.

In order to determine the role of the Arp2/3 complex in formation and/or growth of the germline ring canals, the Arp2/3 subunit, ArpC2 was depleted from the germline using the UAS/GAL4 system. To achieve a strong depletion, the maternal triple driver (*otu-GAL4; nanos-GAL4; nanos-GAL4*) was crossed to the UAS-ArpC2-RNAi line, and female progeny from this cross were incubated for 48 hours at 29°C prior to dissection. Depletion of ArpC2 consistently altered the size of the ring canals compared to controls. Interestingly, there was an increase in the size of the ring canals at stage 7, whereas there was a significant decrease in the size of the ring canals from stage 8-10b (Figure 1, $p < 0.05$, $n \geq 33$). Depletion of ArpC2 did not affect the localization of the ring canal protein, Hts-RC; however, the actin looked clouded around the ring canals. (Figure 1B).

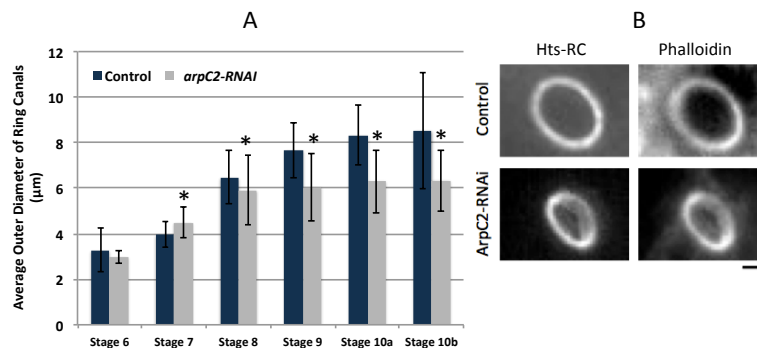


Figure 1. Ring canals in Arp2/3 depleted egg chambers are smaller than control egg chambers. Prior to dissection, *arpC2-RNAi x otu-GAL4; nanos-GAL4; nanos-GAL4* flies along with the control, *w1118*, flies were put through a 48 hour incubation period at 29°C. The control, *w1118*, flies were crossed to the same driver as the treatment flies. (A) Average outer diameter of ring canals between nurse cells during stages 6-10b of oogenesis. (B) Stage 10a egg chamber ring canals. Scale bar is 10 μm. Similar results are seen in the four ring canals between nurse cell and oocyte (data not shown). The asterisk indicated statistically significant data ($p < 0.05$). At each stage $n \geq 33$.

The strength of an RNAi-based depletion using the UAS/GAL4 system in

Drosophila can be altered by changing the temperature or time of incubation prior to

dissection. In order to get a stronger phenotype, the incubation time for the *arpC2-RNAi* egg chambers was increased to 72 hours at 29°C, keeping all other conditions the same as the previous 48 hour incubation. Following this stronger depletion, there was now a consistently significant difference in the diameter of the ring canals at all stages measured from stage 7-

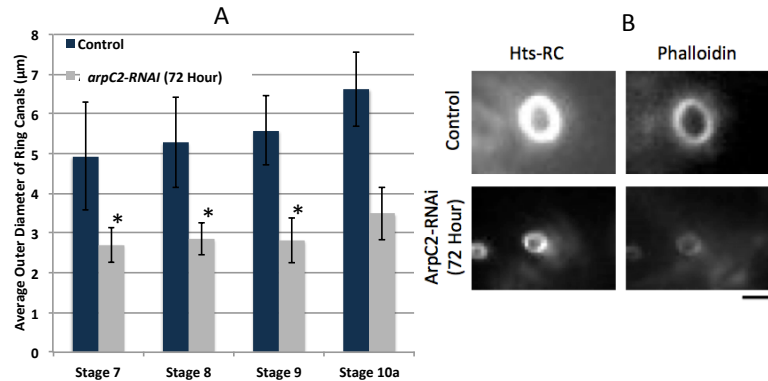


Figure 2. Ring canals in Arp2/3 depleted egg chambers are smaller than controls. Prior to dissection, *arpC2-RNAi x otu-GAL4; nanos-GAL4; nanos-GAL4* flies along with the control, *w1118*, flies were put through a 72 hour incubation period at 29°C. The control, *w1118*, flies were crossed to the same driver as the treatment flies. (A) Average outer diameter of ring canals between nurse cells during stages 7-10a of oogenesis. (B) Stage 9 egg chamber ring canals. Scale bar is 10 μm. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). The asterisk indicated statistically significant data ($p < 0.05$). For all stages $n \geq 22$.

10a (Figure 2, $p < 0.05$, $n \geq 22$), with smaller ring canals than the 48 hour time point at stage 6-9 (Figure 3, $p < 0.05$, $n \geq 22$). This suggests that the larger diameter observed at stage 7 in the 48 hour timepoint could represent a weaker Arp2/3 depletion phenotype.

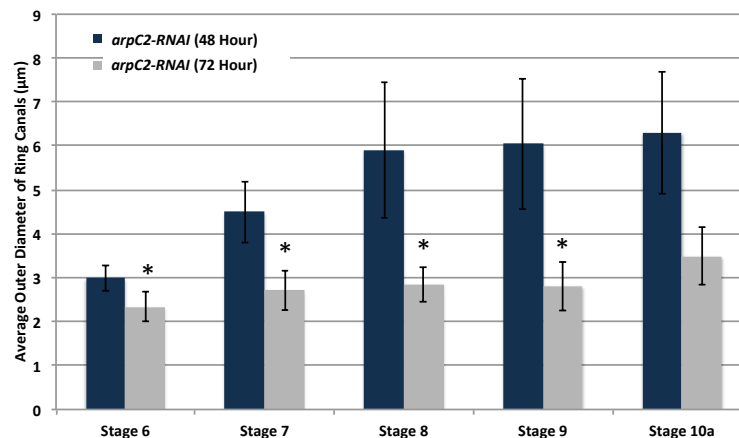
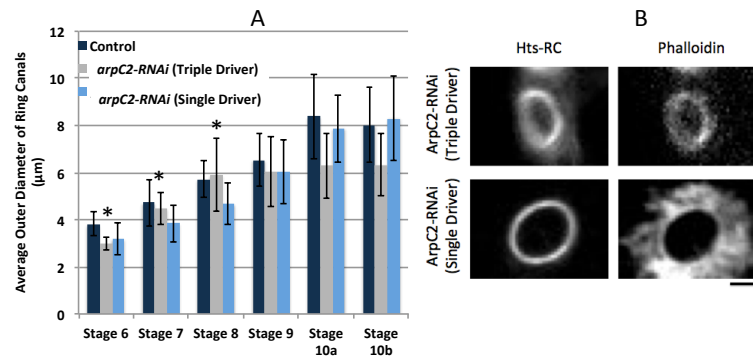


Figure 3. A longer incubation period for the Arp2/3 depletion treatment led to a more severe decrease in ring canal size. Ring canals in *arpC2RNAi x otu-GAL4; nanos-GAL4; nanos-GAL4* egg chambers with 72 hour incubation are consistently smaller than those with 48 hour incubation. Prior to dissection, flies were put through a 48 and 72 hour incubation period at 29°C. Average outer diameter of ring canals between nurse cells during stages 6-10a of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). The asterisk indicated statistically significant data ($p < 0.05$). For all stages $n \geq 22$.

Another tool that is available to characterize the Arpc2 depletion phenotype is a single nanos-GAL4 germline driver (*nos-GAL4*). Using a different driver allowed us to investigate the range of phenotypes that could be generated by Arpc2 depletion. Depletion of Arpc2 using this single GAL4 driver incubated for 48 hours at 29°C led to significantly smaller ring



canals from stages 6-8 (Figure 4; $p < 0.05$, $n \geq 55$). Stages 9-10b are not significantly different in size from the control; however, collapse was seen in the later stages, suggesting that stability is affected (Figure 4,5). Therefore, this suggests that strong depletion of Arpc2 disrupts the normal growth of the ring canals that occurs during stages 6-10; however, weaker depletion of Arpc2 could lead to expansion of ring canals compared to controls, as was observed at stage 7 (Fig. 1).

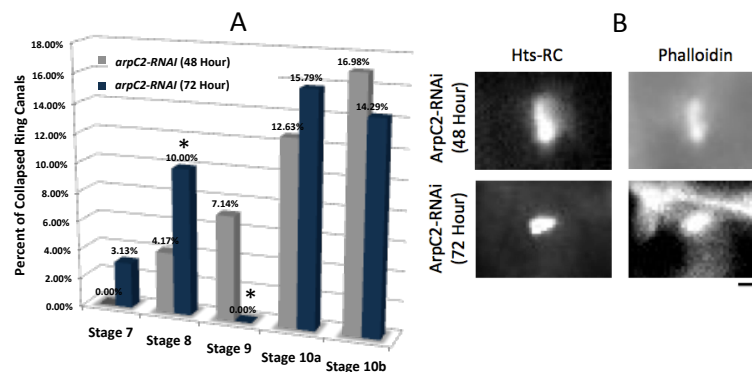


Figure 5. Depletion of the Arp2/3 protein complex can lead to ring canal collapse. (A) Percent was calculated, taking the total number of collapsed or missing ring canals compared to total number of ring canals between nurse cells during stages 7-10b of oogenesis. The asterisk indicated statistically significant data ($p < 0.05$). At each stage $n \geq 22$. (B) Stage 9 egg chamber collapsed ring canals. Scale bar is 10 μm.

Ring Canal Collapse

In addition to changes in ring canal size, depletion of ArpC2 also led to ring canal collapse or disorganization. Therefore, in addition to measuring ring canal size, the number of collapsed or missing ring canals were quantified. No collapse was seen in control egg chambers, as expected. Missing or collapsed ring canals were seen at both the 48 hour and 72 hour incubation using the triple driver (Figure 5, $p < 0.05$, $n \geq 22$). The number of collapsed or missing ring canals also increases over the course of oogenesis. This suggests that depletion of ArpC2 not only alters ring canal growth, but also stability, the effect of which becomes more pronounced as the egg chambers approach nurse cell dumping.

Mutation of Arp2/3 complex members also alters ring canal size.

In order to confirm that the depletion phenotype is caused by reducing levels of Arp2/3 complex members, germline mosaic clones were generated using Arp2/3 complex member loss-of-function mutations.

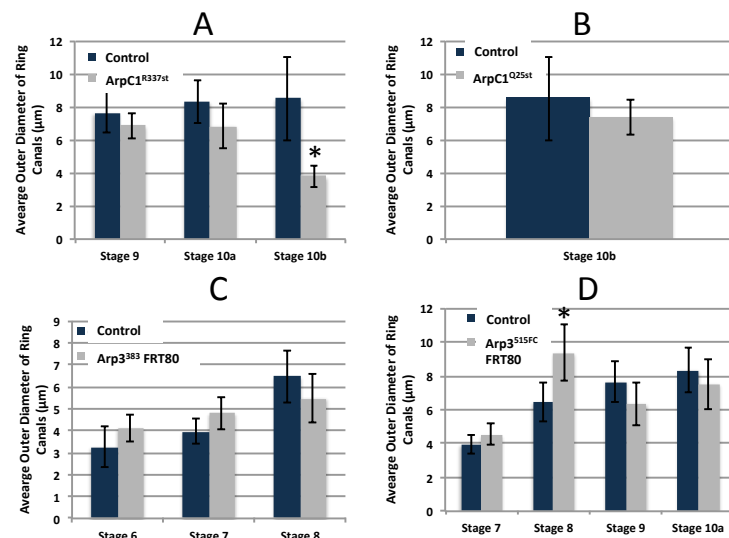


Figure 6. Ring canals in mosaic germline at varying stages of development. Average outer diameter of ring canals between nurse cells during stages 6-10b of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). The asterisk indicated statistically significant data ($p < 0.05$). All stages had $n \geq 10$.

Germline mosaic clones were generated that contained homozygous mutant cells in the

germline of the following mutations: *Arp3*^{515FC} *FRT80*, *Arp3*³⁸³ *FRT80*, *ArpC1*^{Q25st} and *ArpC1*^{R337st} (Figure 6A-D). For each mutant cross, the adult female offspring were dissected after 48 hours on yeast at 25°C, and only egg chambers that contained homozygous mutant clones were imaged. There was no significant difference between mutant ring canal size and control ring canals in two of the four mutant treatments (Figure 6). Although the clone number was very low, for the *ArpC1*^{R337st} mosaic clones, there was a difference in ring canal size at stage 10b (Figure 6A, p<0.05, n=10). In the *Arp3*^{515FC}, there was a significant difference in ring canal size at stage 8 (Figure 6D, p<0.05, n=11). The ring canals at this stage were significantly larger than the control ring canals, and were larger than the ring canal size of the mutants at all other stages. Ring canal collapse was evident in the *Arp3*³⁸³ *FRT80* and the *ArpC1*^{R337st} mutants, suggesting that mutations in the Arp2/3 protein complex can lead to loss of stability in ring canals (Figure 7). Although the mutant data are not as strong as the RNAi data, they do suggest that the RNAi phenotype is likely specific to reducing Arp2/3 complex levels.

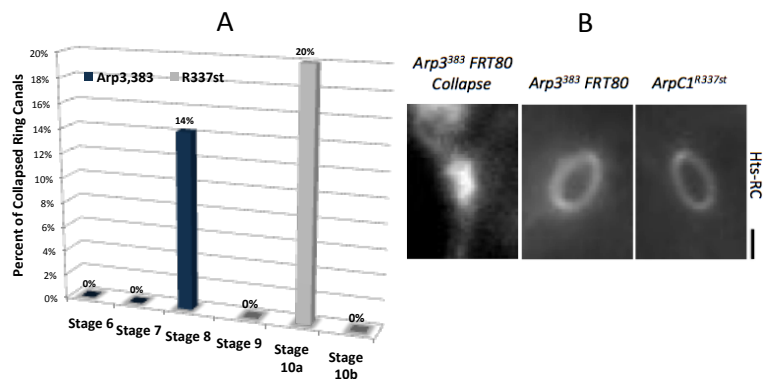


Figure 7. Mutation of the Arp2/3 protein complex can lead to ring canal collapse. (A) Percent of collapsed ring canals between mutant egg chambers, *Arp3*³⁸³ *FRT80* and *ArpC1*^{R337st}. Percent was calculated, taking the total number of collapsed or missing ring canals compared to total number of ring canals between nurse cells during stages 6-10b of oogenesis. (B) Stage 8 egg chamber ring canals for *ArpC1*^{R337st} and stage 9 egg chamber ring canals for *Arp3*³⁸³ *FRT80*. Scale bar is 10 μm.

Acute inhibition of Arp2/3 function leads to decrease in ring canal size.

Mutation or depletion of the Arp2/3 complex is induced early in oogenesis, so these approaches do not address whether Arp2/3 complex activity is continuously required during oogenesis to maintain ring canal structure or promote ring canal growth. To determine if Arp2/3 activity is

continually required, the compound CK-666 was

used in a 120 minute chemical inhibition of Arp2/3 on w^{1118} egg chambers cultured *ex vivo*. The average ring canal size in the CK-666-treated egg chambers was significantly

smaller than the control, CK-689, at every stage measured (5-10b) (Figure 8, $p < 0.05$, $n \geq 29$). Additionally, the ring canals in the CK-666 treated egg chambers were much thicker

(visualized using Hts-RC

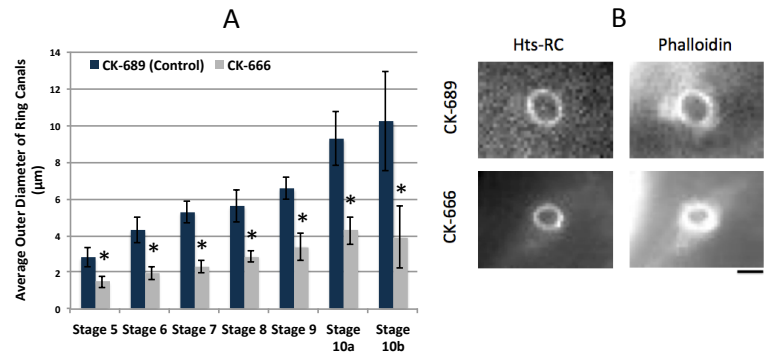


Figure 8. Egg chambers treated with the Arp2/3 inhibitor have smaller ring canals compared to controls. (A) Ring canals were treated with Arp2/3 inhibitor drug, CK-666. Controls were treated with control drug, CK-689. Average outer diameter of ring canals between nurse cells during stages 5-10b of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). The asterisk indicated statistically significant data ($p < 0.05$). All stages had a $n \geq 29$. (B) Stage 10b egg chamber ring canals. Scale bar is 10 μm.

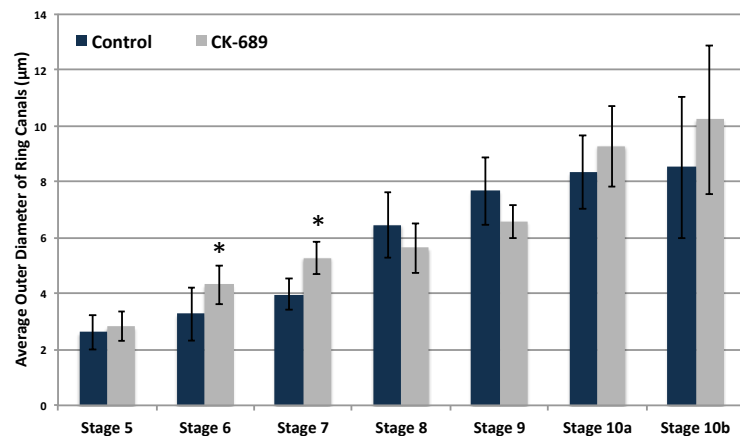


Figure 9. Ring canals in egg chambers treated with control drug, CK-689, are similar to control egg chambers. Average outer diameter of ring canals between nurse cells during stages 5-10b of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). The asterisk indicated statistically significant data ($p < 0.05$). All stages had a $n \geq 29$.

stain) than after treatment with CK-689 (Figure 8B). To insure that the solvent used to dissolve the drug and control molecules, DMSO, did not alter ring canal size, ring canal size in egg chambers incubated in the control compound were compared to ring canal size in untreated, w^{1118} , flies. There was no significant difference in ring canal size between the CK-689 treated and w^{1118} egg chambers, except at stages 6 and 7 (Figure 9, $n \geq 29$). Although statistically different than the w^{1118} control at stages 6 and 7, the CK-689 control ring canals are larger which supports the assumption that CK-689 or the DMSO solvent is not causing the decrease in ring canal size. Ring canal collapse was evident in the CK-666 treated egg chambers at stage 10b, displaying the loss of stability in ring canals in later stages of oogenesis (Figure 10). Ring canal growth and collapse data suggests that the Arp2/3 protein complex is continually needed throughout oogenesis in order to maintain ring canal growth and stability, and that stage 10 egg chambers are especially sensitive to the acute inhibition of Arp2/3 activity.

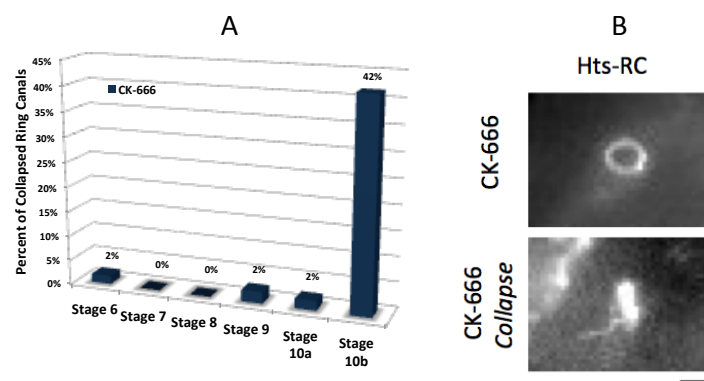


Figure 10. Percent of collapsed ring canals in CK-666 treated egg chambers. Percent was calculated, taking the total number of collapsed or missing ring canals compared to total number of ring canals between nurse cells during stages 6-10b of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). (B) Stage 10b egg chamber ring canals. Scale bar is 10 μ m.

Msn and Arp2/3 promote ring canal growth.

Although it has been shown that the Arp2/3 complex is required to promote the

normal growth and stability of the germline ring canals, little is known about how the Arp2/3 complex is locally activated during oogenesis. Recent work has shown that the Ste20 kinase, NIK, phosphorylates and activates the Arp2/3 complex (16). The homolog of the NIK kinase is Misshapen (Msn), which also localizes to the germline ring canals

(data not shown). When Msn was depleted from the germline using the triple driver, ring canals were significantly larger than the control at stage 6, but smaller than the control at stages 8-10a (Figure 11, $p < 0.05$, $n \geq 42$),

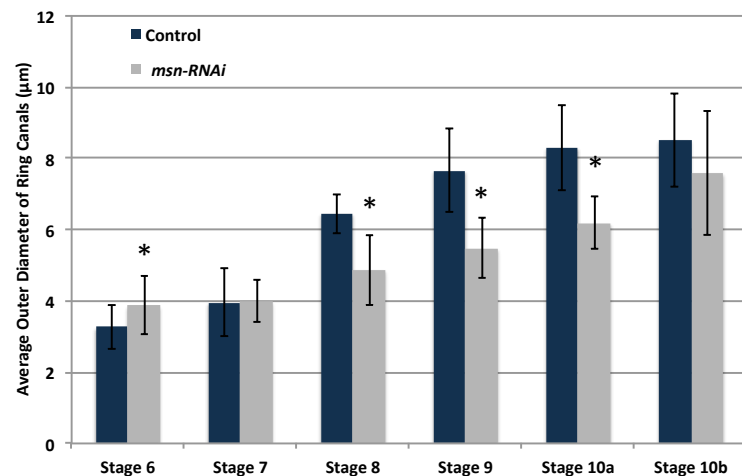


Figure 11 . Ring canals Msn-depleted egg chambers are smaller than controls. Prior to dissection, all flies were put through a 48 hour incubation period at 29°C. Average outer diameter of ring canals between nurse cells during stages 6-10b of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (data not shown). The asterisk indicated statistically significant data ($p < 0.05$). All stages have a $n \geq 42$.

which is similar to phenotype observed in the *arpC2-RNAi* condition (Figure 1,2). The similarity in the phenotypes caused by depletion of Msn or ArpC2 suggests that the two could function in the same pathway to promote ring canal growth.

To test whether Msn and ArpC2 could be functioning in the same pathway, egg chambers were co-depleted of both proteins in the germline. Co-depletion of both Msn and ArpC2 led to significantly larger ring canal size than just the *arpC2-RNAi* at stages 9-10b (Figure 12, $p < 0.05$, $n \geq 33$). However, at stage 8 egg chambers co-depleted of both proteins showed a significantly smaller ring canal size compared to depletion of ArpC2 alone (Figure 14, $p < 0.05$, $n \geq 33$). The *msn-RNAi* & *arpC2-RNAi* egg chambers showed a

larger average ring canal size than *msn-RNAi* egg chambers at stages 8,9 and 10a, and a smaller ring canal size than *msn-RNAi* at stage 6 (Figure 12, $p < 0.05$, $n \geq 33$). These data suggest that Msn and Arp2/3 could work together in the germline; however additional experiments must be conducted in order to determine how the two are functioning to promote ring canal growth and stability.

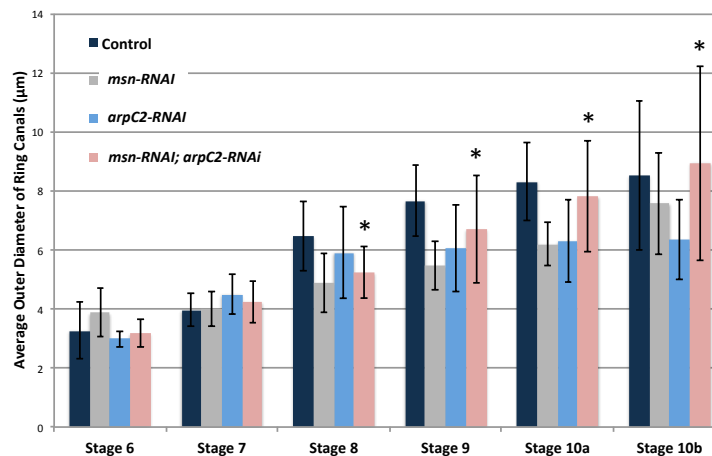


Figure 12. Msn and Arp2/3 could function together in a pathway to promote ring canal development. Ring canals in *msn-RNAi*; *ArpC2-RNAi* \times *otu-GAL4*; *nanos-GAL4*; *nanos-GAL4* egg chambers are larger than ring canals in *msn-RNAi* egg chambers and *arpC2-RNAi* egg chambers in later stages. Prior to dissection, flies were put through a 48 hour incubation period at 29°C. Average outer diameter of ring canals between nurse cells during stages 6-10b of oogenesis. Similar results are seen in the four ring canals between nurse cell and oocyte (*data not shown*). The asterisk indicates statistically significant data between the *msn-RNAi*; *arpC2-RNAi* and the *arpC2-RNAi* treatments ($p < 0.05$). All stages have a $n \geq 33$. Same Control and Msn-RNAi data included from Figure 13.

Discussion

Intercellular bridge function is important for proper formation of germ cells in many organisms. In the developing fruit fly egg, the germline intercellular bridges, or ring canals, are essential for proper oocyte development. The Arp2/3 protein complex localizes to the ring canal and understanding its role in ring canal structure and function will be essential to our understanding of intercellular bridge function and fertility in higher organisms (13). Altering the levels and activity of the Arp2/3 complex led to changes in ring canal size, growth, and stability, suggesting the Arp2/3 protein complex has an essential role in intercellular bridge function (Figure 1,7,8,10,12).

The Arp2/3 protein complex is required for ring canal growth.

Reducing the levels of the Arp2/3 complex significantly changed the size of the germline ring canals. When the Arp2/3 protein complex was depleted through RNA interference, the diameter of the ring canals was significantly smaller through stages 7-10b compared to controls (Figure 1, $p < 0.05$, $n \geq 33$; Figure 4, $p < 0.05$, $n \geq 22$), and the actin looked clouded (Figure 1B). The depletion phenotype was verified through the use of loss-of-function mutations, which showed a decrease in ring canal size in later stages of oogenesis (Figure 6A, $p < 0.05$, $n = 10$; Figure 7). This suggests that the protein complex plays an integral role in supporting ring canal growth, which is consistent with previous work utilizing Arp2/3 mutants (3). This analysis was focused on stages 6-10, in which the size of the ring canals is expanding significantly, and it did not test whether reducing Arp2/3 levels would alter the size or structure of the ring canals prior to stage 6.

Interestingly, acute inhibition of the Arp2/3 complex with CK-666 led to a significant decrease in the size of the ring canals compared with egg chambers treated with the control molecule CK-689 from stages 5-10b (Figure 8, $p < 0.05$, $n \geq 29$). The small molecule inhibitor, CK-666, binds to the Arp2/3 protein complex and inhibits actin nucleation, leaving the complex inactive (19). My data would suggest that Arp2/3 activity is continuously required to maintain the expanded state of the ring canals, and that inhibition compromises the structure and leads to reduction. Additional experiments will be necessary to determine whether Arp2/3 complex activity is continuously required during all stages of oogenesis or just during the later stages that were measured (5-10).

In order to accommodate the movement of nutrients from the nurse cells to the oocyte, ring canals must grow in size significantly (from $\sim 0.5 \mu\text{m}$ in diameter to $\sim 10 \mu\text{m}$ in diameter; 11). As the ring canals grow there is a change in the organization of actin filaments in the intracellular bridges (12). Early in ring canal expansion, the number of actin filaments increases to around 700 filaments and the diameter increases to about $5 \mu\text{m}$. However, around stage 5 of oogenesis, the number of filaments remains constant while the diameter continues to expand (3), which suggests that the primary period of Arp2/3 activity could be prior to stage 5 when the number of actin filaments is increasing. When the Arp2/3 protein complex is altered through mutation, depletion, or inhibition, changes in ring canal size and collapse occurs. Depletion or mutation of the Arp2/3 complex could lead to decreased filament number in this initial stage of expansion through stage 5. Fewer actin filaments present in the ring canal during this

initial phase of expansion could prevent the later expansion observed in the subsequent stages (6-10). Conversely, even if the net number of actin filaments is not changing, it is possible that there is assembly and disassembly of actin filaments occurring during the later stage of expansion to promote this growth.

Interestingly, acute inhibition of later stage egg chambers (stages 6-10) caused a significant and consistent effect on ring canals size. This suggests that the Arp2/3 complex is continuously required to maintain the expanded state of the ring canals. Because these stages of oogenesis are ~3-6 hours in duration (22), in the egg chambers evaluated, the acute inhibition of the Arp2/3 complex would only alter Arp2/3 activity during stages ~5-10. Therefore, this shows that Arp2/3 activity is necessary during these stages, even though the number of actin filaments is not changing significantly. In the different Arp2/3 conditions, the ring canals have thinner actin staining that becomes more clouded as oogenesis increases (Figure 1,2,4,5,8). The Arp2/3 complex, when working properly, would help maintain the structure of the ring canal, which has been described as a net of bundled actin (8); however, when absent or inhibited, the actin becomes fuzzy and the ring canals collapse (13). Additional experiments must be performed in order to further evaluate the role for the Arp2/3 complex in maintaining the structure and organization of the actin filaments within the ring canals. For example, electron microscopy could be utilized in order to evaluate the organization and packing of actin filaments in egg chambers treated with CK-666.

Results from the mutation, depletion, and inhibition of the Arp2/3 protein complex show that loss of Arp2/3 function leads to ring canals collapse (Figure 5,7, and 10). Ring canals show the highest level of collapse in later stages of oogenesis among treatments (Figure 10). The collapse of these compromised ring canals in later stages of development could be due to the stress that is building on the expanded ring canals as they approach nurse cell dumping. It could also be due to cortical tension that the structures would be feeling due to actin-myosin based contractility. The nurse cell dumping rates transition from slow to fast, helping the nutrient flow from nurse cell to oocyte (21). If the structure of the ring canal is compromised, then it may be unable to withstand these stresses and ultimately collapse, leading to the formation of a multinucleate nurse cell. This is seen in the CK-666 treated egg chambers (Figure 10).

Arp2/3 plays a role in ring canal development along with Msn.

Preliminary data has demonstrated that altering the levels of the Msn kinase leads to similar effects on ring canal size and structure as the Arp2/3 complex (Figure 12). Interestingly, the homolog of Msn, NIK, was recently shown to phosphorylate and activate the Arp2/3 complex (16). When Msn is depleted from the germline using RNAi with a triple maternal GAL4 driver, ring canals were larger than controls at stage 7 and then smaller at stages 8-10a (Figure 11, $p < 0.05$, $n \geq 42$). This suggests that Msn is necessary for ring canal growth in later stages. Additionally, the *msn-RNAi* condition showed ring canal collapse, suggesting that Msn also plays a role in ring canal stability. These data suggest that both Msn and Arp2/3 may work in the same pathway in order to promote ring canal growth and stability.

In order to determine if both Msn and Arp2/3 are working within the same pathway, both proteins (Msn and Arp2/3) were depleted using RNA interference. Compared to controls, depletion of both protein complexes showed significant differences from controls at stages 7-9 (Figure 12, $p < 0.05$, $n \geq 33$). Ring canals for the double RNAi egg chambers were smaller than controls during later stages (stages 7-10b); however, they were larger than egg chambers from the single RNAi treatment group from stages 8-10b. When performing a double RNAi depletion such as *msn-RNAi* & *arpC2-RNAi*, there is a chance that the depletion of each one is weaker than seen in a single RNAi condition. Having seen stronger phenotypes at longer incubation stages for single RNAi conditions, a stronger depletion can be tested for in the future by using a 72 hour incubation period for the double RNAi condition, or combinations of mutations and RNAi depletions could be performed. Although there is a possibility that the depletion is weaker in the double RNAi condition, results showed that the double RNAi condition had statistically different ring canal sizes compared to both single RNAi conditions (Figure 12, $p < 0.05$, $n \geq 33$). Both single RNAi conditions resulted in smaller ring canal sizes in later stages than in the double RNAi condition, suggesting that depletion of both is leading to a partial rescue of the phenotype, with similar ring canal size to control egg chambers (Figure 12, $p < 0.05$, $n \geq 33$). Since ring canal size in older co-depleted egg chambers seems to be relatively normal, there may be another pathway compensating for the loss of these two regulators. In the future, more studies evaluating co-depletion of Arp2/3 and Msn as well as other ring canal proteins can be done to evaluate the connection between Arp2/3, Msn, and other protein complexes localizing at the ring

canals that may play a role in the pathway. To better support the need for the Arp2/3 protein complex throughout all stages of oogenesis, future studies done should also evaluate the sizes of ring canals at earlier stages of development.

Conclusion

Using *Drosophila melanogaster* as a model system, this study evaluated the role for the Arp2/3 complex in intercellular bridge structure and growth. Using loss-of-function mutations and RNAi-based depletions in the germline, it was evident that the Arp2/3 protein complex is required for ring canal growth and stability. Further, acute inhibition of Arp2/3 function using the CK-666 inhibitor revealed that the Arp2/3 complex is continually required for ring canal stability throughout oogenesis.

Despite this essential role for the Arp2/3 complex in the germline, the pathway by which the Arp2/3 complex maintains ring canal stability and promotes growth is unclear. Data from inhibiting both the Msn and Arp2/3 complexes suggest that Msn and Arp2/3 may work together. However the pathway is uncertain and results suggest that there may be other influences that compensate for the loss of both Msn and Arp2/3. Further research is required; however, these initial results are promising. Overall, these findings support the original hypothesis that the Arp2/3 protein complex is required for intercellular bridge growth and development continually through development. Learning more about Arp2/3 role in ring canal stability and development along with its regulators can provide valuable information in the study of gametogenesis and infertility across many organisms.

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